The kinetics of dissociation of the inhibitor of nucleoside transport, nitrobenzylthioinosine, from the high-affinity binding sites of cultured hamster cells

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Nucleoside transport in various types of animal cells is inhibited by the binding of nitrobenzylthioinosine (NBMPR) to a set of high-affinity sites on the plasma membrane. This work examined the binding of [^3H]NBMPR to the nucleoside transporters of cultured Nil 8 hamster fibroblasts and of cells of a virus-transformed clone (Nil SV) derived from Nil 8. Experiments conducted with intact Nil 8 and Nil SV cells and with membrane preparations indicated that the two lines differed significantly in the cellular content of binding sites and only slightly in the affinities of these sites for NBMPR. Nil 8 and Nil SV cells possessed $(4.2\text{--}8.0) \times 10^5$ and $(2.0\text{--}4.0) \times 10^6$ sites per cell respectively, whereas the dissociation constants of site-bound NBMPR obtained with intact cells and with membrane preparations were similar, ranging from 0.29 to 1.5 nM. Dilazep, a potent inhibitor of nucleoside transport that is structurally unrelated to NBMPR, appeared to compete with NBMPR for binding to the high-affinity sites when tested under equilibrium conditions with K_i values for inhibition of NBMPR binding to Nil 8 and Nil SV cells respectively of 15 ± 4 and 32 ± 4 nM. The dissociation of NBMPR from the binding site–NBMPR complex of Nil SV membrane preparations was a first-order decay process with a rate constant of $0.68 \pm 0.26 \text{ min}^{-1}$. The rate of dissociation of NBMPR from the binding-site complex of membrane preparations and intact cells was decreased significantly in the presence of dilazep and increased in the presence of the permeant uridine. These results suggest that the apparent competitive-inhibition kinetics obtained for dilazep under equilibrium conditions should not be interpreted as binding of dilazep to the same site as NBMPR but rather as binding of the two inhibitors to closely associated sites on the nucleoside transporter. Similarly, uridine also appears to bind to a site separate from the NBMPR-binding site.

NBMPR and related S^6 -derivatives of 6-thiopurine nucleosides are potent inhibitors of nucleoside transport in animal cells (Paterson & Oliver, 1971; Paterson *et al.*, 1977, 1981). The reversible, specific interaction of NBMPR with transport-inhibitory sites in the plasma membrane was established in

studies with human erythrocytes of the relationship between high-affinity binding of NBMPR and inhibition of nucleoside transport by NBMPR (Pickard *et al.*, 1973; Cass *et al.*, 1974). Fractional occupancy by NBMPR of the binding sites (about 10^4 per erythrocyte; K_D about 1 nM) was proportional to inhibition of equilibrium exchange diffusion of uridine (Cass *et al.*, 1974). A correlation between the cellular capacity (V_{max}) for zero-*trans* influx of uridine and the number of cellular NBMPR-binding sites has been shown for erythrocytes from several different species (Jarvis & Young, 1980; Jarvis *et al.*, 1982). These findings indicate that the nucleoside-transport capacities (turnover rates) of the nucleoside-transporter ele-

Abbreviations used: NBMPR, 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; dilazep, 3,4,5-trimethoxybenzoic diester with tetrahydro-1*H*-1,4-diazepine-1,4(5*H*)-dipropanol; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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ments are similar in erythrocytes of different species and that the reported differences between those cell types in nucleoside-transport capacity are attributable to differences in membrane content of functional nucleoside-transport elements.

Two well known inhibitors of nucleoside transport, dipyridamole and dilazep, although structurally unrelated to NBMPR, inhibit binding of NBMPR to its binding sites (Pohl & Brock, 1974; Paterson *et al.*, 1980, 1983). When tested under equilibrium binding conditions, dipyridamole and a variety of permeant nucleosides inhibited NBMPR binding to erythrocytes (Jarvis & Young, 1982) and HeLa cells (Paterson *et al.*, 1980) in an apparently competitive manner. {The concentration of dilazep that reduced by 50% the site-specific binding of [G^3H]NBMPR to cultured S49 mouse lymphoma cells was 25–30 nM (E. S. Jakobs, P. M. Roth and A. R. P. Paterson, unpublished work).} The evidence so far described is in agreement with a simple model in which permeant nucleosides, NBMPR and the structurally unrelated inhibitors interact with the nucleoside transporter at a single class of sites. In this connection, it is noteworthy that the high-affinity membrane sites at which NBMPR binds in effecting inhibition of nucleoside transport are evidently of a single type in erythrocytes (Cass *et al.*, 1974; Jarvis & Young, 1980) and in cultured cells (Lauzon & Paterson, 1977; Cass *et al.*, 1981).

Although the apparently competitive inhibitions noted above suggest a simple model in which inhibitor and permeant nucleoside compete at the permeation site of the nucleoside transporter, other evidence suggests that a more complex model may be required. Some cultured cells have a component of nucleoside permeation that is not inhibited at nanomolar concentrations of NBMPR [HeLa cells (Dahlig-Harley *et al.*, 1981), leukaemia L1210 cells (Belt, 1983), Novikoff hepatoma cells (Wohlhueter *et al.*, 1978), yet is clearly mediated in the hepatoma cells (Plagemann & Wohlhueter, 1980)].

Characterization of transport systems with low sensitivity to NBMPR is at an early stage. The 'NBMPR-insensitive' component of uridine permeation in HeLa cells is abolished by concentrations of *p*-chloromercuribenzenesulphonate that have little effect on the NBMPR-sensitive component (Dahlig-Harley *et al.*, 1981), suggesting, perhaps, that NBMPR and permeant-nucleoside-binding sites may be physically distinct from each other, but functionally coupled by a mechanism that involves the *p*-chloromercuribenzenesulphonate-sensitive groups. It has been shown in erythrocytes that 25 mM-uridine enhances, rather than inhibits, the binding of the fluorescent analogue of NBMPR, dansylaminoethylmercaptoguanosine (Shohami & Koren, 1979), a further indication that the permeant-nucleoside- and inhibitor-binding sites may be distinct.

The present study attempted to test the idea that the permeant uridine might interact with sites on the nucleoside transporter that are distinct from those that bind the inhibitors of nucleoside transport, NBMPR and dilazep. We used Nil 8 cells, a permanent, cloned line derived from a culture of golden hamster embryo cells, and Nil SV, a virus-transformed clone derived from Nil 8 cells. It was reported previously (Heichal *et al.*, 1979) that Nil 8 and Nil SV cells exhibited uridine-transport activities that were partially sensitive to NBMPR. The reported kinetic characteristics of uridine transport in Nil 8 and Nil SV cells may be compared as follows: (a) K_m values for uridine influx were similar (516 ± 199 and $400 \pm 64 \mu M$ respectively), but (b) V_{max} values differed (3600 ± 1170 and 1500 ± 170 pmol/min per 10^6 cells respectively). In the present paper we describe the kinetics of [3H]NBMPR dissociation from membrane binding sites on cells of the two hamster lines. These studies assumed (a) that, in the presence of a large (1000-fold) excess of non-radioactive NBMPR, association of [3H]NBMPR with its binding site was negligible, and (b) that rates of dissociation of [3H]NBMPR would be unaffected by the presence of compounds that bind only to the same sites as [3H]NBMPR. Experiments are reported that indicate that the kinetics of dissociation of bound [3H]NBMPR are influenced by the permeant uridine and the inhibitor dilazep which is structurally unrelated to NBMPR. These observations provide evidence for separate classes of sites for permeants and inhibitors despite the apparently competitive relationships between the latter seen in experiments conducted under equilibrium conditions.

Materials and methods

Cell culture

Cells used in this study were Nil 8, a cloned line of golden hamster fibroblasts, and Nil SV, a transformed clone derived from Nil 8 cells by infection with murine sarcoma virus (Heichal *et al.*, 1979). Cultures were started from frozen stocks of cells at 6-week intervals, and were maintained at 37°C in humidified air containing 5% CO₂. Both cell lines were subcultured twice weekly in 75-cm² plastic culture flasks (Nunc Intermed, Roskilde, Denmark) by plating 2×10^6 cells in 10 ml of Dulbecco's modified Eagle's medium containing 10% foetal-calf serum and 50 units each of penicillin and streptomycin/ml.

Binding of NBMPR to intact cells

For assays of NBMPR binding, replicate cultures in 35-mm plastic Petri dishes (Nunc Intermed, Roskilde, Denmark) were started with cells at densities between 1×10^5 and 5×10^5 cells/dish and when used in experiments (24–96 h after seeding)

these cultures contained $(0.8\text{--}1.6) \times 10^6$ cells. In binding assays, growth medium from such cultures was replaced with 500 μl of Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1974) containing [^3H]NBMPR at graded concentrations with or without 5 μM -non-radioactive NBMPR. Incubation intervals of 20 min were allowed for the attainment of equilibrium between free and cell-bound NBMPR during which cultures were kept on a metal tray in a shaking water bath at 37°C. Binding intervals were ended by removal of the incubation medium, which was reserved for assay of ^3H content. Cultures were then rinsed individually five times with 2 ml portions of ice-cold PBS (this procedure was completed within 15 s). The cells in each dish were extracted with 750 μl of 5% Triton X-100 for 20 min at room temperature, and 500 μl portions of each extraction mixture were assayed for ^3H content by liquid-scintillation counting (Pande, 1976). Samples (100 μl) of the incubation medium were assayed for ^3H radioactivity to determine remaining concentrations of unbound NBMPR. Sums of experimentally determined bound and free NBMPR in assay mixtures were within 5% of the total NBMPR added in each assay.

Binding of NBMPR to crude membrane fractions

Cells from drained 24–96-h cultures in 75-cm² flasks were scraped into ice-cold PBS/5 (PBS diluted with 4 vol. of water) to achieve a final suspension of about 2×10^7 cells in 5 ml of PBS/5. The suspensions were homogenized (20 strokes) in a tight-fitting Dounce homogenizer, with cell breakage confirmed by phase-contrast microscopy. The homogenates were centrifuged at 4°C (121 g for 10 min), the pellets were discarded and the supernatant fractions were then re-centrifuged at 4°C (12000 g for 20 min). The pellet fractions, which contained membranes, were resuspended in volumes of PBS/5 calculated to contain cell material derived from 0.5×10^6 cells in each 50 μl portion, the volume used in assays of NBMPR binding. Pellet suspensions (crude membrane preparations) were kept at 4°C until used in binding assays. The protein content of these preparations was determined by the Coomassie Blue binding assay of Bradford (1976) using reagents from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Binding reactions were started by adding 50 μl portions of membrane suspension to plastic tubes containing [^3H]NBMPR and other components of the assay in 250 μl of PBS. Assay mixtures for the determination of non-specific binding contained non-radioactive 5 μM -NBMPR. During intervals of incubation, tubes were kept in a shaking bath at 37°C. To end binding reactions, assay mixtures were rapidly diluted with 3 ml of ice-cold PBS; dilution was followed by immediate fast filtration

through GF/C 2.4-cm glass microfibre filters (Whatman, Clifton, NJ, U.S.A.) and three fast washes with 3 ml portions of ice-cold PBS (the filters had been prewashed with 1 ml of unlabelled 5 μM -NBMPR solution and 3 ml of PBS). A filtering manifold was employed for the filtering and washing procedures, which were completed within 10 s. Filters were then placed in scintillation vials and assayed for ^3H content after at least 2 h of incubation in a Triton-based scintillation fluid (Pande, 1976). To determine concentrations of free [^3H]NBMPR, reaction mixtures were sampled just before the terminating dilution step. The 150 μl samples were placed in chilled centrifuge tubes, spun at room temperature (12000 g for 5 min) in an Eppendorf model 5412 centrifuge and supernatants were assayed for ^3H content. Sums of measured free and bound NBMPR accounted within experimental error for the [^3H]NBMPR added to reaction mixtures.

Rates of dissociation of the NBMPR-binding-site complex

Intact cells. Time courses for dissociation of NBMPR from its binding sites on intact cells were obtained by measuring cellular retention of ^3H after exposure of replicate [^3H]NBMPR-labelled cultures to isotope-free medium. Cultures were first labelled by equilibration with [^3H]NBMPR-containing medium for 20 min and, to initiate timed intervals for dissociation of the [^3H]NBMPR-binding-site complex, the equilibration medium was replaced with PBS containing 5 μM -NBMPR or binding inhibitors under study. Dissociation intervals were terminated with cold rinsings of cells as described above, and cells were assayed for ^3H content. It was determined in a control experiment that rates of NBMPR dissociation from the ligand-binding-site complex were the same whether the initial exposure of cells to [^3H]NBMPR was for 1 or 20 min.

Membranes. Two procedures were used to measure kinetics of dissociation of [^3H]NBMPR from binding sites on membrane preparations at 37°C. For both, membranes, prepared as in the procedure described above, were labelled by a 20 min exposure to [^3H]NBMPR, as in the assay of NBMPR binding to cells.

(a) 100-Fold dilution. The dissociation process was initiated by combining replicate 100 μl samples of mixtures of labelled membranes with 10 ml portions of PBS with or without unlabelled NBMPR (or inhibitors of NBMPR binding) and was ended by removing samples at timed intervals and filtering as described above.

(b) Isotope dilution. In this procedure, replicate 300 μl samples of labelled membrane mixtures were mixed with 100 μl portions of PBS with or without unlabelled NBMPR (or binding inhibitors) to initiate the dissociation process, and, after graded intervals,

dissociation was terminated by filtration of individual reaction mixtures.

For both (a) and (b), the ^3H content of membrane material was determined as described above for studies of $[^3\text{H}]\text{NBMPR}$ binding to membrane preparations.

Incorporation of thymidine into the acid-insoluble fraction of cells

When cultures were incubated with $500\ \mu\text{l}$ of PBS containing $0.5\ \mu\text{M}$ - $[^3\text{H}]\text{thymidine}$ ($0.5\ \mu\text{Ci/ml}$), the 1 h incubation intervals were terminated and monolayers were washed as in binding experiments with intact cells. The washed monolayers were extracted for 20 min with 5% trichloroacetic acid at 4°C and the acid-insoluble residues remaining on the culture dishes were then rinsed twice with the same reagent. The acid-insoluble residues were dissolved in $600\ \mu\text{l}$ of $0.5\ \text{M}$ -KOH for 30 min at room temperature. Samples of the latter solutions and of the acid extract were assayed for ^3H content by liquid-scintillation counting.

Chemicals

Cell-culture materials were obtained from GIBCO Canada, Burlington, Ont., Canada. $[\text{G}-^3\text{H}]\text{NBMPR}$ was purchased from Moravsek Biochemicals, Brea, CA, U.S.A. NBMPR was prepared (Paul *et al.*, 1975) using as starting material 6-thioinosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Dilazep was a gift from F. Hoffmann-La Roche & Co., Basel, Switzerland.

Data analysis

Site-specific and non-specific binding of NBMPR. Data obtained for the equilibrium binding of $[^3\text{H}]\text{NBMPR}$ were first corrected for non-specific binding and then subjected to mass-law analysis either by the Scatchard method (Edsall & Wyman, 1958) or by means of a hyperbolic curve-fitting procedure using the Cleland (1967) algorithm. Both procedures produced identical binding parameters, and the data were consistent with simple binding to a homogeneous population of binding sites. A direct proportionality between non-specifically bound NBMPR and the equilibrium concentration of free NBMPR was demonstrated. Non-specific binding ($[^3\text{H}]\text{NBMPR}$ that associated with samples in the presence of $5\ \mu\text{M}$ -NBMPR) was measured in all binding experiments and was applied as a correction to total binding in proportion to equilibrium concentrations of free NBMPR. Non-specific binding did not exceed 30% of total binding and was less than 10% in most cases, both for intact cells and membrane preparations. Because a significant fraction of non-specifically bound $[^3\text{H}]\text{NBMPR}$ was associated with the plastic culture dishes or filters,

cell concentrations (and protein concentrations in experiments with the membrane preparations) were kept as large as possible to minimize the fraction of total binding attributable to non-specific binding.

Data are reported throughout with standard deviations from the mean.

Inhibition of binding by compounds other than NBMPR. In the analysis of binding data from experiments in which site-specific binding of $[^3\text{H}]\text{NBMPR}$ was inhibited by other compounds, the following equation was used to evaluate K_i , the apparent dissociation constant of the non-radioactive inhibitor at NBMPR-binding sites.

$$K_{\text{Dapp}} = K_{\text{D}} \left(1 + \frac{[\text{I}]}{K_i} \right) \quad (1)$$

In this relationship, which assumes competition between $[^3\text{H}]\text{NBMPR}$ and its inhibitor, K_{D} is the dissociation constant of NBMPR from its binding site in the absence of the competing inhibitor, and $[\text{I}]$ is the concentration of free inhibitor, which, as a first approximation, was set equal to the initial inhibitor concentration. This approximation is not valid at low concentrations of tight-binding inhibitors if concentrations of free inhibitor are significantly reduced by binding depletion.

In the analysis of data of Fig. 3, we have used the following simple relationship:

$$K_{\text{Dapp}} = \frac{f(q-b)}{b} \quad (2)$$

In this relationship, K_{Dapp} is the apparent dissociation constant for site-bound $[^3\text{H}]\text{NBMPR}$, f is the concentration of free $[^3\text{H}]\text{NBMPR}$, q is the total concentration of binding sites, b is the concentration of sites occupied by $[^3\text{H}]\text{NBMPR}$, and $q-b$ is the concentration of binding sites unoccupied by $[^3\text{H}]\text{NBMPR}$. By rearranging this equation, one obtains the following relationship, in which $R = b/f$.

$$K_{\text{Dapp}} = q/R - f \quad (3)$$

If the total concentration of sites, q , is known, one may judge the validity of the assumption of competitive inhibition by a non-radioactive agent, I , by determining f and b at different concentrations of I and by plotting values of $q/R - f$ as a function of I . If the assumption is valid, such plots should be straight lines because q , the total number of binding sites available to NBMPR, would not be affected by the presence of a competitive inhibitor. Furthermore, such plots should be coincident at different concentrations of NBMPR.

Kinetics of NBMPR dissociation from the NBMPR-binding-site complex. All binding-complex-dissociation data were treated as first-order decay reactions either by plots of $\ln(A - A_\infty)$ versus t , where A is the amount of NBMPR bound at time t and A_∞ is the amount bound at $t = \infty$, or by using

a non-linear exponential fit. Both approaches yielded the same rate constants.

Results

Results of experiments that measured equilibrium binding of [³H]NBMPR to Nil 8 and Nil SV cells from proliferating and non-proliferating cultures are presented in Fig. 1 and Table 1. Although the affinity of NBMPR for its binding sites was slightly greater for Nil 8 than for Nil SV cells, the most striking difference between the two cell lines was the total number of sites available for high-affinity binding of NBMPR. The number of sites per cell was at least 4-fold greater in the transformed than in the non-transformed cells. This difference appeared to be independent of the state of growth of the cells since it was similar in experiments conducted at 1, 4 and 5 days after cells were plated when the levels of DNA synthesis in the two cell lines were both high, high and low or both low.

Equilibrium binding experiments similar to those conducted with intact cells were performed with crude membrane fractions prepared from growing and non-growing Nil 8 and Nil SV cells. The results are presented in Fig. 2 and Table 2. The concentration of NBMPR-binding sites in the crude membrane fraction from Nil SV cells was much higher than in the corresponding fraction from Nil 8 cells whether the membrane fractions were prepared

Table 1. Binding of NBMPR to Nil 8 and Nil SV cells from proliferating and non-proliferating cultures
Binding of [³H]NBMPR to intact cells was measured as described in the Materials and methods section. The cultures used in Expt. 1 were initiated at 1 × 10⁶ cells/dish; those used for Expts. 2 and 3 at 0.2 × 10⁶ cells/dish. The results of Expt. 2 are represented graphically in Fig. 1.

Expt.	Age of culture (days)	Cell line	DNA synthesis (pmol/10 ⁶ cells)	K _D (nM)	10 ⁻⁵ × Number of sites per cell
1	1	Nil 8	24.6	0.40 ± 0.03	5.6 ± 0.2
		Nil SV	26.6	1.21 ± 0.20	22.0 ± 4.0
2	4	Nil 8	6.3	0.29 ± 0.05	4.2 ± 0.4
		Nil SV	34.0	0.66 ± 0.03	24.0 ± 0.1
3	5	Nil 8	3.0	0.35 ± 0.01	8.0 ± 0.2
		Nil SV	1.0	1.02 ± 0.20	40.0 ± 0.1

Fig. 2. Binding of NBMPR to membranes prepared from Nil 8 to Nil SV cells

Binding of [³H]NBMPR to membrane preparations from 1-day cultures of Nil 8 (a) and Nil SV (b) was measured under equilibrium conditions as described in the Materials and methods section. Results are presented as Scatchard plots and are summarized in Table 2 (Expt. 1).

Fig. 1. Binding of NBMPR to Nil 8 and Nil SV cells
Binding of [³H]NBMPR to Nil 8 (○) and Nil SV (●) cells was measured under equilibrium conditions (20 min, 37°C) as described in the Materials and methods section. Cultures were used 96 h after plating and contained 0.78 × 10⁶ cells/dish (Nil 8) and 0.95 × 10⁶ cells/dish (Nil SV). Results are presented as direct binding isotherms and as Scatchard plots (inset) and are summarized in Table 1 (Expt. 2).

Table 2. Binding of NBMPR to membrane fractions prepared from Nil 8 and Nil SV cells from proliferating and non-proliferating cultures

Binding of [³H]NBMPR to membrane preparations was measured as described in the Materials and methods section. The results from studies with 1-day cultures (Expt. 1) are represented graphically in Fig. 2.

Expt.	Age of culture (days)	Cell line	K _D (nM)	Sites (fmol/μg of protein)	Protein (μg/assay)
1	1	Nil 8	1.50 ± 0.20	12.7 ± 1.2	7.5
		Nil SV	0.54 ± 0.03	42.8 ± 1.6	9.5
	3	Nil 8	0.91 ± 0.07	8.6 ± 0.4	11.6
		Nil SV	0.66 ± 0.10	79.0 ± 10.1	16.5
2	4	Nil 8	0.88 ± 0.09	10.5 ± 0.1	13.0
		Nil SV	2.02 ± 0.40	63.8 ± 1.6	16.6

Fig. 3. The effect of dilazep on binding of NBMPR to Nil 8 and Nil SV cells

Binding of [^3H]NBMPR to 4-day cultures of intact Nil 8 (1.3×10^6 cells/dish) (a) and Nil SV (1.6×10^6 cells/dish) (b) was measured as described in the Materials and methods section. Cultures were incubated for 20 min in PBS containing 2 nM- (O) or 10 nM- (Δ) [^3H]NBMPR in the absence or in the presence of dilazep at the concentrations indicated. Non-specific binding of [^3H]NBMPR and the equilibrium concentrations of [^3H]NBMPR were determined as described under 'Data analysis'. The values for q that were used in the analysis were calculated from the numbers of sites per cell determined in Expt. 2, Table 1, and were, for Nil 8 and Nil SV cells respectively, 1.15 nM and 3.8 nM.

Fig. 4. The effect of dilazep on the apparent affinity of NBMPR for binding to intact Nil SV cells

Binding of [^3H]NBMPR to 3-day cultures of intact Nil SV cells (0.9×10^6 cells/dish) was measured in the presence ($\bullet$) and in the absence (O) of 100 nM-dilazep under equilibrium conditions as described in the Materials and methods section. Results are presented as Scatchard plots and the number of binding sites and apparent K_D values obtained in the presence and absence respectively of dilazep were 4.15 ± 0.55 nM and 7530 ± 67 fmol/ 10^6 cells and 1.2 ± 0.2 nM and 7870 ± 1000 fmol/ 10^6 cells.

from 1-day sparse cultures (growing) or from 4-day confluent cultures (non-growing). The ratio of the binding-site densities in the two types of intact cells was similar to the ratio of binding-site densities in crude membrane preparations from the two cell types.

Dilazep, an inhibitor of adenosine transport (Pohl & Brock, 1974; Paterson *et al.*, 1983), was shown to be a potent inhibitor of NBMPR binding to Nil 8 and Nil SV cells. The influence of dilazep on the equilibrium binding of NBMPR to high-affinity sites on Nil 8 and Nil SV cells was evaluated in the experiment of Fig. 3. Data for the site-specific binding of [^3H]NBMPR in the absence and presence of graded dilazep concentrations were analysed by eqn. (1) (see the Materials and methods section) using the K_D values for NBMPR determined in Expt. 2, Table 1. The inhibition constants (K_i) for the inhibition by dilazep of NBMPR binding to Nil 8 and Nil SV cells respectively were 15 ± 4 and 32 ± 4 nM. In Fig. 3, values of the function, $q/R - f$ (equivalent to $K_{D,app}$; see eqn. 3), are plotted against dilazep concentration. The linear relationship evident in these plots is consistent with the assumption of competition under equilibrium conditions between dilazep and NBMPR at the binding sites. It may be noted that the data obtained at two

different NBMPR concentrations, 2 and 10 nM, are approximately coincident (see eqn. 3 commentary).

In the experiment of Fig. 4, site-specific binding of graded concentrations of NBMPR to intact Nil SV cells was measured in the presence and absence of 100 nM-dilazep. It is clear in these data that the presence of dilazep altered the apparent affinity of NBMPR for the binding sites. The intersection of Scatchard plots on the horizontal axis (bound NBMPR) indicates that the maximum number of sites available to NBMPR in the presence of dilazep was the same as that available in the absence of dilazep, giving the appearance of competition between the ligand and the inhibitor for the same binding sites.

With the membrane-containing crude cell fractions, such as those used in the experiment of Fig. 2, it was possible to measure the dissociation of the [^3H]NBMPR-membrane complex under conditions of high (100-fold) dilution. Such large dilutions were not practical with the monolayer cultures. Fig. 5 illustrates the kinetics of dissociation of the NBMPR-membrane complex caused by a 100-fold dilution of the reaction mixture. Whereas dilution into PBS alone caused only partial dissociation of the complex, dilution into PBS containing non-radioactive

Fig. 5. The kinetics of dissociation of $[^3\text{H}]\text{NBMPR}$ from membranes prepared from Nil SV cells

Membrane preparations, obtained from Nil SV cells as described in the Materials and methods section, were first incubated ($133\mu\text{g}$ of protein/ml) for 20 min at 37°C in the presence of 7.5 nM $[^3\text{H}]\text{NBMPR}$, and the dissociation process was then followed under conditions described for the 100-fold dilution procedure. Time courses of dissociation of $[^3\text{H}]\text{NBMPR}$ were determined in PBS containing 0 ($\circ$), 5 (Δ), 54 ($\diamond$) (results presented in the inset only), and 540 ($\bullet$) nM non-radioactive NBMPR. Theoretical curves are shown as continuous lines. The theoretical curve in the presence of NBMPR was drawn using a non-linear curve-fitting procedure for an exponential decay process and yielded a rate constant, k , of 0.77 ± 0.03 . The theoretical curve in the absence of NBMPR ($\circ$) was drawn assuming two processes, using the equation $A = B_1e^{-k_1t} + B_2e^{-k_2t}$ and these parameters: $B_1 = 2670\text{ c.p.m.}$, $B_2 = 1130\text{ c.p.m.}$, $k_1 = 1.04\text{ min}^{-1}$, $k_2 = 0.014\text{ min}^{-1}$. In the inset, results are plotted as $\ln(A - A_\infty)$, where A and A_∞ are the amounts of $[^3\text{H}]\text{NBMPR}$ bound respectively at times t and ∞ .

NBMPR (5–540 nM) resulted in complete dissociation of the NBMPR–binding-site complex. The data obtained by diluting the NBMPR–membrane complex into a large volume of PBS appeared to represent the sum of two separate processes. One of these was fast, with a rate constant similar to that obtained in the presence of NBMPR, and accounted for partial dissociation of the complex, and the other process, which was slow, would eventually lead to complete dissociation of the complex. The experiment of Fig. 5 indicated that the presence of NBMPR in the diluting medium did not

Fig. 6. The effect of dilazep on the kinetics of dissociation of NBMPR from intact Nil SV cells

Cultures of Nil SV cells (0.9×10^6 cells/dish) were incubated for 20 min at 37°C in the presence of 2.6 nM $[^3\text{H}]\text{NBMPR}$. The dissociation process was initiated at zero time by addition of PBS containing $5\mu\text{M}$ NBMPR ($\circ$), $25\mu\text{M}$ dilazep (Δ) or $5\mu\text{M}$ NBMPR and $25\mu\text{M}$ dilazep ($\bullet$). The theoretical curves were obtained from the data plotted by a non-linear curve-fitting procedure for an exponential decay process. The rate constants so obtained are presented in Table 3.

affect the rate of dissociation of $[^3\text{H}]\text{NBMPR}$ from the complex since time courses for the fast component of the dissociation process were similar in the presence and in the absence of NBMPR. When pooled NBMPR-binding data (corrected for loss via the slow component of dissociation) were analysed in a logarithmic replot (Fig. 5, inset), it was evident that the fast component of dissociation could be described as a single first-order decay process with a rate constant of $0.68 \pm 0.26\text{ min}^{-1}$.

Fig. 6 demonstrates that dilazep altered the rate of dissociation of NBMPR from binding sites on intact Nil SV cells, a result that is inconsistent with simple competition between dilazep and NBMPR at the binding sites. After cells were labelled by incubation for 20 min with 2.6 nM $[^3\text{H}]\text{NBMPR}$ in PBS, the dissociation process was initiated by replacing the labelling medium with PBS containing $5\mu\text{M}$ NBMPR, $25\mu\text{M}$ dilazep or both together. The dissociation rate in the presence of dilazep alone was significantly lower than the rate observed in the presence of non-radioactive $5\mu\text{M}$ NBMPR. When both dilazep and non-radioactive NBMPR were added simultaneously, the kinetic pattern of dissociation was the same as that obtained with dilazep alone. Dilazep also altered the kinetic pattern of dissociation of $[^3\text{H}]\text{NBMPR}$ from Nil 8 cells and from membranes prepared from Nil SV cells (Table 3).

Table 3. *The effect of dilazep on the kinetics of dissociation of NBMPR*

Experiments with intact cells and with membrane preparations were performed as described in the legends to Figs. 5 and 6. The rate constants presented below were obtained by fitting an exponential decay equation to the data using a non-linear curve-fitting procedure.

Type of binding experiment	Dissociation medium		Rate constant (min ⁻¹)
	[NBMPR] (nM)	[Dilazep] (μM)	
Intact Nil SV cells*	5	0	0.48 ± 0.02
	0	25	0.12 ± 0.01
	5	25	0.18 ± 0.01
Intact Nil 8 cells	5	0	0.76 ± 0.09
	0	25	0.11 ± 0.01
	5	25	0.10 ± 0.01
Membranes prepared from Nil SV cells	5	0	0.68 ± 0.07
	0	25	0.16 ± 0.02
	5	25	0.12 ± 0.01

* These results are also presented in Fig. 6.

The experiments of Fig. 7 and Table 4 evaluated the influence of uridine on the dissociation of NBMPR from binding sites on Nil SV cells. In contrast with the inhibitory influence of dilazep on the dissociation rate of NBMPR from the NBMPR-binding-site complex, that rate was increased in the presence of uridine. NBMPR dissociation was not complete in the presence of uridine, and a new equilibrium level of extracellular free NBMPR was reached (Fig. 7). The rate constant describing the approach to the new equilibrium was $0.51 \pm 0.05 \text{ min}^{-1}$. The rate constant for dissociation of [³H]NBMPR from Nil SV cells in the presence of non-radioactive NBMPR was $0.39 \pm 0.04 \text{ min}^{-1}$, whereas the rate constant for dissociation in the presence of both non-radioactive NBMPR and uridine was $0.68 \pm 0.08 \text{ min}^{-1}$. These results indicate that uridine accelerated dissociation of [³H]NBMPR from its binding sites.

In the experiment of Fig. 7, only minor dissociation of the [³H]NBMPR-binding-site complex resulted when the [³H]NBMPR labelling medium was replaced with PBS. This result is not inconsistent with the higher dissociation rates seen when labelled membrane preparations were diluted 100-fold, as in Fig. 5. In the experiment of Fig. 7, because the volume of replacement medium was small, a significant fraction of the total [³H]NBMPR present in the initial reaction mixture was bound to cells, and a new equilibrium between the dissociated (free) NBMPR and the remaining bound NBMPR was soon reached.

Fig. 7. *The effect of uridine on the kinetics of dissociation of NBMPR from intact Nil SV cells*

Cultures of Nil SV cells (1.3×10^6 cells/dish) were incubated for 20 min at 37°C in the presence of 1.3 nM [³H]NBMPR. Dissociation kinetics were measured as described in the legend to Fig. 6 in the absence (O) or in the presence of 20 mM-uridine (Δ), non-radioactive $5 \mu\text{M}$ -NBMPR (◇), or 20 mM-uridine and non-radioactive $5 \mu\text{M}$ -NBMPR (●). The theoretical curves were drawn using a non-linear curve-fitting procedure for an exponential decay process and the rate constants obtained are presented in Table 4.

Table 4. *The effect of uridine on the kinetics of dissociation of NBMPR*

Experiments with intact Nil SV cells and with membrane preparations were performed as described in the legends to Figs. 5–7. Rate constants were obtained as described in Table 3.

Binding to:	Dissociation medium		Rate constant (min ⁻¹)
	[NBMPR] (μM)	[Uridine] (mM)	
Intact cells	5	0	0.39 ± 0.04*
	0	20	0.51 ± 0.05*
	0	40	0.62 ± 0.06
	5	20	0.68 ± 0.08*
	5	40	0.71 ± 0.01
Membranes	5	0	0.82 ± 0.05
	0	20	1.08 ± 0.11
	0	40	1.73 ± 0.08
	5	20	1.60 ± 0.07
	5	40	1.48 ± 0.05

* The curves from which these constants were derived are presented in Fig. 7.

Discussion

The results in Fig. 1 and Table 1 indicate that, although the binding constant for NBMPR on Nil 8 and Nil SV cells differed only by a factor of

2, NBMPR binding sites were several times more abundant on transformed cells than on the non-transformed cells. This difference was not due to differences between the two cell types in cell volume or shape, nor to differences in NBMPR uptake and metabolism by the intact cells, since it was also seen in experiments with membrane preparations. It was shown previously (Heichal *et al.*, 1979) that the maximum rate of uridine transport by Nil 8 cells is more than twice that of Nil SV cells. These findings can be interpreted in two different ways: (a) the number of NBMPR-binding sites cannot be equated with the number of functional transporters in Nil 8 and Nil SV cells; (b) the turnover number (transport capacity) of nucleoside transporters of Nil SV cells is lower than that of Nil 8 transporters. Although we cannot distinguish between these two possibilities, each suggests that the simple correlations recognized previously for red blood cells between NBMPR-binding sites and functional nucleoside-transport elements (Cass *et al.*, 1974; Jarvis & Young, 1980; Jarvis *et al.*, 1982) do not hold for these cultured cells.

With membrane preparations, we studied the dissociation of the NBMPR-binding site complex after 100-fold dilution of the ligand concentration in the suspending medium under conditions that should, at equilibrium, have resulted in less than 16% occupancy of NBMPR binding sites assuming K_D values for the NBMPR-site complex of Nil SV membranes of 0.5 to 2 nM (Table 2). In the experiment of Fig. 5, dilution alone resulted in 70% dissociation of the complex. The time-course data obtained were not fit by a first-order kinetic equation, but were better described by two such equations, raising the possibility that NBMPR-binding sites of more than one class were present in the preparation. Whereas dilution alone resulted in only partial dissociation of the [^3H]NBMPR-binding site complex, complete dissociation took place when the diluent contained non-radioactive 5 nM-NBMPR (Fig. 5). This result also suggests binding site heterogeneity and perhaps the existence of sites that must undergo conformational changes before ligand dissociation may occur. Although the latter interpretation would imply co-operativity in the binding of NBMPR to its sites, no such co-operative effects were observed in the Scatchard analysis of binding under equilibrium conditions.

Figs. 6 and 7 and Tables 3 and 4 describe the kinetic pattern of NBMPR dissociation from the binding-site complex in the presence of (a) large excesses of non-radioactive NBMPR, (b) the transport inhibitor dilazep or (c) the permeant uridine. The observed rate of dissociation of the [^3H]NBMPR-membrane complex in the presence of dilazep alone, or of dilazep plus NBMPR, was significantly less than in the presence of NBMPR alone. This result

indicates that dilazep interacted with the entity containing the bound NBMPR and appears to exclude the possibility that NBMPR and dilazep bind to the same physical site. In the presence of 25 μM -dilazep, any re-association of the binding-site complex was highly improbable. The same interpretation may be made of the observed accelerating effect of uridine on the rate of dissociation of NBMPR from its binding sites (Fig. 7, Table 4). Enhancement of the rate of NBMPR dissociation in the presence of uridine suggests that the non-radioactive ligand affected in some way the binding of NBMPR to its site.

In conclusion, the results reported here suggest that, despite the apparently competitive nature of their inhibition of NBMPR binding under equilibrium conditions, uridine (Jarvis & Young, 1980) and dilazep (Figs. 3 and 4) may not bind to the same site as NBMPR. Dilazep and uridine may affect rates of dissociation of NBMPR by shifting an equilibrium between two states of the binding site that differ in their affinities for NBMPR or by changing the rate of interconversion between these two states. This interpretation implies that the nucleoside transporter has at least one allosteric site and argues against a simple one-site model for NBMPR inhibition of the nucleoside-transport system.

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